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Prothymosin a (PTa) is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. Its high conservation in mammals and wide tissue distribution suggest an essential biological role. While the exact mechanism of action of PTa remains elusive, the one constant has been its relationship with the proliferative state of the cell and its requirement for cellular growth and survival. Recently PTa was found to promote transcriptional activity by sequestering the anticoactivator, REA from the Estrogen Receptor (ER) complex. We now report that Estradiol (E2) upregulates PTa mRNA and protein expression. Further studies indicate that ERa regulates PTa gene transcriptional activity. We have also delimited the region of PTa gene promoter involved in ERa mediated transcriptional regulation and identified a novel ERa-binding element. Increased intracellular PTa expression in the presence of estrogen is accompanied by increased nuclear/decreased cytoplasmic localization. Increased nuclear expression of PTa in correlated with increased proliferation as measured by expression of Ki67 nuclear antigen. Conversely, inhibition of nuclear PTa expression in breast cancer cells using antisense methodology resulted in the inhibition of E2-induced breast cancer cell proliferation. Overall these studies underscore the importance of PTa in estrogen-induced breast cell proliferation.

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INTRODUCTION

The proposed studies in this research grant focused on a Estrogen Receptor (ER) upregulated gene, Prothymosin α (PTα). PTα is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. Its high conservation in mammals and wide tissue distribution suggest an essential biological role. While the exact mechanism of action of PTα remains elusive, the one constant has been its relationship with the proliferative state of the cell and its requirement for cellular growth and survival. Recently PTα was found to promote transcriptional activity by sequestering the anticoactivator, REA from the Estrogen Receptor (ER) complex. We observed that Estradiol (E_2) upregulates PTα mRNA and protein expression. Further studies indicate that ERα regulates PTα gene transcriptional activity. The specific aims proposed in this grant addressed the functional relevance of PTα in breast cancer gene expression and proliferation and the mechanism of regulation of PTα by the ER.

BODY

We have completed Task 1 and about 70% of Task 2 of the original proposal. These studies also resulted in the attached publication in the journal Oncogene. Some of our findings are described in the enclosed manuscript published in the journal Oncogene (Bianco and Montano, 2002). Major accomplishments described in this publication include (1) mutational analyses to further delimit the fragment required for Estrogen Receptor (ER) binding to and transcriptional activation of the PTα gene promoter (2) further verification of the role of PTαin estrogen-induced breast epithelial cell proliferation using quantitative double immunocytochemistry.

The role of PTα as a transcriptional regulator and other cellular function can be accomplished by further characterizing the PTα-interacting clones we have identified in the yeast two hybrid screenings. Sequence of one of the putative PTα-interacting clones indicates that its identity is Elongation Factor 1 β (EF1β). This factor is involved in the proposed link between translational control and cell growth regulatory pathways. We verified the interaction of EF1β with PTα using *in vitro* glutathione-S-transferase (GST)-pull down assays). The affinity column for these assays consisted of PTα expressed as a fusion protein with GST bound to a Glutathione-Sepharose beads. *In vitro* translated and radiolabeled EF1β was retained in the column indicating a direct interaction between EF1β and PTα. EF1β was not retained in a column consisting only of GST.

On the last year of funding we have redone the yeast two hybrid screenings and have identified another putative PTα-interacting clone. The interaction was verified in vitro using GST-pull down assays. The clone encodes a protein, CGI-48, of unknown function. Further studies are necessary to determine the functional implications of the interaction of PTα with clones isolated from yeast two hybrid screenings

As an alternative and also to verify findings from yeast two hybrid screenings we proposed to use mass spectrometry technology. A proteomics core facility in Cleveland Clinic is available to further characterize the multiprotein complex wherein PTα exist. It is likely that associated proteins are an integral part of the function of PTα and will be a key to understanding their biological function. We proposed to use conventional chromatography to reduce non-specific proteins by first enriching target proteins prior to

imunoaffinity purification. We will identify associated proteins using antibody-affinity chromatography. We have an antibody for PTα for use in affinity chromatography. After purification of protein complexes, the identity of associated proteins will be determined by mass spectrometry technology. A limiting factor in this experiment has been the quality of our PTα antibody. On the last year of funding we found that it may not be of sufficient quality for affinity chromatography as we were not able to immunoprecipitate. Thus further optimization of our immunoprecipiation procedure is required or another antibody may have to be generated.

Please note that we did not feel the need to examine the effectiveness of PTα antibody injections in inhibiting PTα activity and compare to antisense methodology as we have been able to inhibit PTα expression using antisense retroviruses. Antibody injections, which are technically very challenging, were proposed as an alterative approach should antisense technology not work. We also did not feel it was necessary to determine whether suppression of PTα expression results in loss of myc-dependent growth of MCF7 cells as our studies indicate that ER regulation of PTα is direct and does not require c-myc.

KEY RESEARCH ACCOMPLISHMENTS

A. The following were accomplished and reported in the Oncogene manuscript.

Task 1. Determined the mechanism of regulation of Prothymosin α gene transcriptional activity by the estrogen receptor (ER)

- constructed reporter constructs containing deletion mutants of the regulatory region of the PTα gene
- examined the activities of deletion mutant reporter constructs in MCF breast cancer cells in transfection assays
- biochemical analyses of the interactions of MCF7 breast cancer cell factors with the PTα gene regulatory regions

Task 2. Determined the functional importance of estrogen-mediated increase in Prothymosin α expression on the mitogenic effects of estrogens in breast cancer cells

- determined time course for maximal induction of PTα expression by estrogens
- demonstrated that antisense retroviruses can inhibition of PTα expression
- determined whether suppressing PTα expression results in partial or complete loss of estrogen-dependent growth of MCF7 cells

B. The following were accomplished but the data were not published:

- identified PTα interacting clones using the yeast two hybrid system
- verified interactions using *in vitro* protein-protein interaction assays

C. The following were not completed:

- examine the effect of inhibition of PTα expression on the induction of reporter activity in MCF7 cells that also contain either serum responsive element (SRE)-, TPA-responsive element (TRE), or cAMP responsive element (CRE)-LacZ indicator plasmids

- further characterize clones isolated from yeast genetic screenings

REPORTABLE OUTCOMES

Bianco NR and **Montano MM**. (2002) Transcriptional regulation of Prothymosin alpha gene by the Estrogen Receptor: Molecular mechanisms and functional implications. Oncogene, 21:5233-5244

CONCLUSIONS

We have shown estrogen-stimulated gene transcription from PTα gene promoter-containing reporter constructs. We have delimited the region required for estrogen-mediated induction to a 43-bp fragment, and the mechanism for ERα-mediated activation most likely involves a complex interplay between ERα and other protein factors bound to this region. The transcriptional activation by estrogens appears to involve ERα binding to the PTα gene promoter. Estrogen treatment also resulted in increased PTα nuclear localization which in turn is correlated with increased cell proliferation. Our studies also indicate that PTα plays a role in E_2-induced proliferation of breast cancer cells.

While the exact details of the mechanism of action of PTα remain elusive, it is clearly involved in the regulation of cell transformation and proliferation. Future studies on the PTα interacting proteins that we have identified should prove useful in further defining the biological role of PTα. The estrogen receptor (ER) is a ligand activated transcription factor and the identification of "primary" ER target genes is imperative for

understanding the basis for the proliferative action of ER in breast cancer cells. There are only a few candidate genes that appear to be under the direct regulation of the ER; much less genes that are associated with cell proliferative activity. PTα is a particularly strong candidate because its expression and intracellular localization appears to be regulated by estrogen, and down-regulation of PTα expression inhibits E_2-induced breast cancer cell proliferation.

PERSONNEL SUPPORTED BY THE GRANT

Principal Investigator: Monica Montano, Ph.D.

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Regulation of prothymosin α by estrogen receptor α: molecular mechanisms and relevance in estrogen-mediated breast cell growth

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Prothymosin α (PTα) is a small highly acidic protein found in the nuclei of virtually all mammalian tissues. Its high conservation in mammals and wide tissue distribution suggest an essential biological role. While the exact mechanism of action of PTα remains elusive, the one constant has been its relationship with the proliferative state of the cell and its requirement for cellular growth and survival. Recently PTα was found to promote transcriptional activity by sequestering the anticoactivator, REA from the Estrogen Receptor (ER) complex. We now report that Estradiol (E_2) upregulates PTα mRNA and protein expression. Further studies indicate that ERα regulates PTα gene transcriptional activity. We have also delimited the region of PTα gene promoter involved in ERα-mediated transcriptional regulation and identified a novel ERα-binding element. Increased intracellular PTα expression in the presence of estrogens is accompanied by increased nuclear/decreased cytoplasmic localization. Increased nuclear expression of PTα is correlated with increased proliferation as measured by expression of Ki67 nuclear antigen. Conversely, inhibition of nuclear PTα expression in breast cancer cells using antisense methodology resulted in the inhibition of E_2-induced breast cancer cell proliferation. Overall these studies underscore the importance of PTα in estrogen-induced breast cell proliferation. *Oncogene* (2002) **21,** 5233–5244. doi:10.1038/sj.onc.1206545

Keywords: estrogen; estrogen receptor α; prothymosin α

Introduction

The estrogen receptor (ER) protein is essential for mediating the actions of estrogen in target tissues. The binding of estrogen initiates a process of receptor activation that includes the high affinity binding of ER to specific DNA sequences, termed estrogen response elements (EREs). The interaction of ER with EREs results in the modulation of specific gene expression, through which the physiological actions of estrogens are manifested (reviewed in Aranda and Pascual, 2001). Estrogens acting via the ER dramatically escalate proliferative and metastatic activity in breast tumor cells, in part via the induction of growth factors, proteases, and basement membrane receptors (reviewed in Russo and Russo, 1998). However, the relative role of the induction of these genes on the proliferative effects of estrogens in breast cancer cells is not well-defined.

Prothymosin α (PTα) is a small highly acidic protein found in the nuclei of virtually all mammalian tissues (Clinton *et al.*, 1991; Gomez-Marquez and Segade, 1988; Goodall *et al.*, 1986; Manrow *et al.*, 1991; Palvimo and Linnala-Kannkunen, 1990; Watts *et al.*, 1989). Its high conservation in mammals and wide tissue distribution suggest an essential biological role. PTα expression correlates well with the proliferative activity of tissues (Eschenfeldt and Berger, 1986; Gomez-Marquez *et al.*, 1989; Rodriguez *et al.*, 1998; Sburlati *et al.*, 1991; Wu *et al.*, 1997). Recently, PTα was shown to be capable of transforming rodent fibroblast cells in a manner similar to Ras, suggesting that PTα may be an important downstream target for inducers of cellular transformation (Orre *et al.*, 2001). In breast cancer PTα appears to have some prognostic value. PTα expression is higher in tumor samples than in normal breast tissue (Tsitsilonis *et al.*, 1998), and the expression levels of PTα can be correlated with the proliferation status and metastatic potential of tumors (Magdalena *et al.*, 2000; Tsitsilonis *et al.*, 1998).

While PTα appears to play a role in cell proliferation, PTα also has an emerging role in the regulation of transcription. Recently Martini *et al.* (2000) reported that PTα is involved in the transcriptional repression by the anticoactivator factor, Repressor of Estrogen Receptor Activity (REA). PTα is able to promote ER transcriptional activity by sequestering REA from the ER complex. In addition the PTα protein has been localized in the nucleus (Clinton *et al.*, 1991; Manrow *et al.*, 1991; Palvimo and Linnala-Kannkunen, 1990; Wu *et al.*, 1997) and studies suggest that PTα binds specifically to linker histone H1 and cooperates in nucleosome assembly (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsou *et al.*, 1998), implicating a putative nuclear function related to chromatin remodeling. A role for PTα in the transcriptional activation process is supported by studies wherein cells

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overexpressing PTα exhibit more active chromatin and higher rates of transcriptional activity than control cells (Karetsou *et al.*, 1998).

The intracellular signaling pathways governing PTα expression are not well-defined. PTα mRNA levels are increased after serum restitution or after stimulation with various mitogens (Pineiro *et al.*, 2000; Zalvide *et al.*, 1992). Thus far, only two transcription factors have been proposed to positively regulate the PTα gene promoter, E2F (Eilers *et al.*, 1991; Szabo *et al.*, 1993) and c-*myc* (Desbarats *et al.*, 1996; Gaubatz *et al.*, 1994). PTα expression has been proposed to be under the direct control of c-*myc* through a DNA element known as the E-box, however other findings do not confirm this observation (Mol *et al.*, 1995). Studies indicate that c-*myc* expression is not necessary for PTα expression (Loidi *et al.*, 1999). It has been reported that the transcription factor elongation factor (E2F) which is involved in the regulation of genes important in DNA replication and cell cycle regulation also activates PTα gene transcriptional activity (Eilers *et al.*, 1991; Szabo *et al.*, 1993). PTα has also been reported to be negatively regulated by p53, further supporting its potential role in proliferation of the cell (Zhao *et al.*, 2000). The PTα protein is phosphorylated although the physiological relevance of this finding is not known (Perez-Estevez *et al.*, 1997; Sburlati *et al.*, 1993).

We report that estrogens upreglate PTα mRNA and protein levels in breast cancer cells. Estradiol upregulates PTα gene transcription and we have delimited a novel region involved in this transcriptional activation. Our results indicate that ERα is part of the transcriptional complex that binds to PTα gene promoter and suggest direct transcriptional regulation of this gene by ERα. PTα induction by estrogens appears to be involved in estrogen induction of breast cancer cell proliferation.

Results

Identification of PTα as an estrogen-regulated gene

Shown in Figure 1a (left panel) is a Northern blot using the human PTα cDNA as a probe. We found that PTα 1.4 kb mRNA is present at 2–3-fold higher levels in estradiol (E_2)-treated cells when compared to control cells 24 h after treatment. No increase in PTα mRNA was observed in the presence of the antiestrogen ICI182,780 (ICI). Upregulation of PTα by E_2 was evident even with cycloheximide pretreatment, suggesting that intervening protein synthesis is not required. The increase is PTα mRNA in response to estrogens is reflected at the protein level wherein we also observe a 2–3-fold increase (Figure 1a, right panel).

Induction of Prothymosin α gene transcriptional activity by estrogens

To determine if estrogen regulation of PTα expression occurs at the transcriptional level a reporter construct containing the 5′ regulatory region of the PTα gene, pPstI PTα CAT (Figure 1b) (Szabo *et al.*, 1993) (Mol *et al.*, 1995), was transfected into an ER positive breast cancer cell line MCF7 (Figure 1c). After introduction of pPstI PTα CAT, cells were treated with increasing concentrations of E_2 and the antiestrogen trans-hydroxytamoxifen (TOT). Analyses of reporter activity reveal a dose dependent increase in PTα gene transcriptional activity in response to E_2 but not TOT (Figure 1c).

The role of ERα in the transcriptional regulation of the PTα gene was examined in two ERα negative cell lines transfected with an expression vector for wild type ERα. A significant increase in transcriptional activity of the pPstI PTα CAT reporter construct in the presence of estradiol (E_2) was observed in human breast cancer MDA-MB-231 cells when cells were cotransfected with an expression vector for ERα (Figure 2a). A slightly higher activation of PTα gene transcriptional activity by estrogens was evident in Hec-1B human endometrial cancer cells when compared to MDA-MB-231 cells. TOT did not activate transcription from the PTα gene reporter construct in any of these cell lines. No increase in the activity of the control pCAT3 promoter vector was observed with E_2 or TOT (Figure 2a).

Identification of PTα gene promoter regions required for activation by estrogens

To determine the promoter region(s) involved in estrogen activation of PTα gene transcriptional activation, a reporter construct containing the 5′ regulatory region of the PTα gene from the *Apa*I site located −0.819 kb from the transcriptional start site, pApaI PTα CAT, was introduced into MCF7, MDA-MB-231, and Hec-1B cells. Specifically we wanted to determine if the E-box located at −1.173 kb of the PTα gene promoter was required for E_2-induced increase in PTα gene transcriptional activity (Figure 1b). Our results indicate that E_2 induced an increase in pApaI PTα CAT activity and the region between −5 and −0.819 kb of the human PTα gene is not required for transcriptional induction by E_2 (Figure 2b). Note that there were no differences in the basal activities of the pPstI PTα CAT and pApaI PTα CAT constructs (data not shown). Similar observations were made in the three cell lines examined. These results suggest that the induction of PTα gene transcriptional activity by E_2 is not mediated through the E-box in the −5 kb promoter region of the PTα gene. Thus it is unlikely that estrogen-stimulated increase in PTα expression is exerted through increased expression of c-*myc* in response to estrogens (Dubik and Shiu, 1988) and binding of c-*myc* to the E-box in this region. Cells transfected with the empty control pCMV vector (lacking the ERα cDNA) did not show an E_2-mediated increase in pApaI PTα CAT activity (Figure 2b). The antiestrogen TOT blocked E_2-mediated induction of both reporters (Figure 2c). These findings suggest a requirement for the ERα in E_2-mediated transcriptional activation of PTα gene activity.

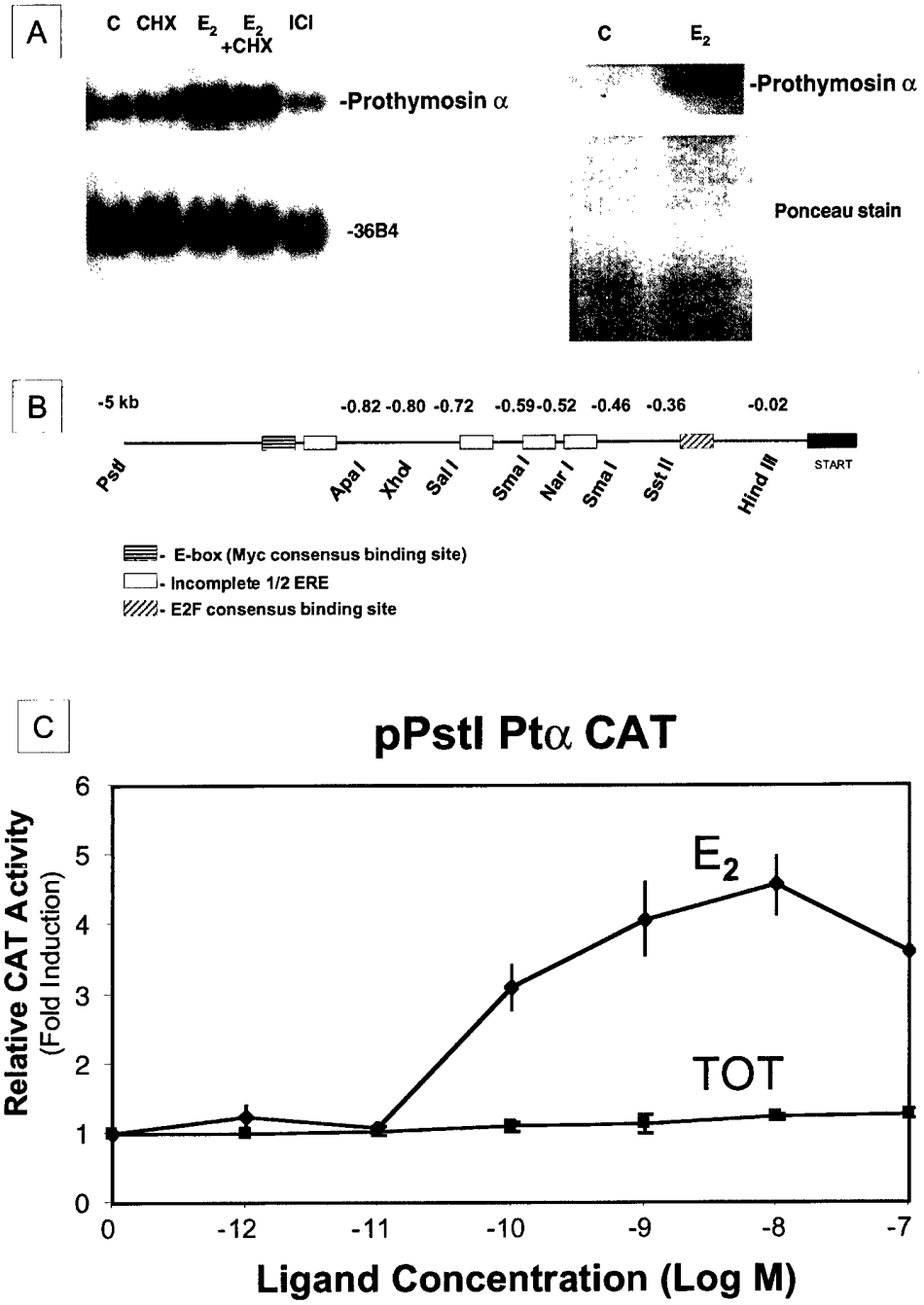

Figure 1 Regulation of PTα gene expression by estrogens at the transcriptional level. (**a**) Left panel; Total RNA was collected from MCF7 parental cells 24 h after treatment with control ethanol vehicle (C), 4 h pretreatment with cycloheximide, (CHX, 10^{-5} M), estradiol (E_2, 10^{-8} M), E_2 (10^{-8} M)+CHX pretreatment, or ICI 182,780 (ICI, 10^{-7} M) as indicated. Equal amounts (20 μg) of total RNA were separated by electrophoresis. The blot was probed with the random primer labeled PTα cDNA. As an RNA loading control, the same blot was reprobed with 36B4 cDNA. Right panel: Western blot analyses of PTα protein levels in MCF7 cells in the absence (C) or presence of E_2. Whole cell lysates were collected 24 h after treatment, electrophoresed on SDS–PAGE gels, transferred to nitrocellulose filters, probed with PTα polyclonal antibody, and visualized using horseradish peroxidase-conjugated secondary antibody. The lower figure shows the Ponceau S-stained blot to show equal loading. The autoradiographs shown in (**a**) are representative of three separate experiments. (**b**) The PTα gene promoter region (**c**) MCF7 cells were transfected with the PTα gene reporter construct, PstI PTα CAT, along with a β-galactosidase internal control reporter to correct for transfection efficiency. Cells were then treated for 24 h with control ethanol vehicle (C) or varying concentrations of E_2 and TOT as indicated. Cell extracts were prepared and analysed for CAT activity and β-galactosidase activity. Values are the means ± s.e.

Deletional analyses of the 819 bp region was performed to further delimit the region of the PTα promoter required for ERα transcriptional activation. These experiments were conducted in Hec-1B endometrial carcinoma cells wherein we observe the highest activation from the pPstI PTα CAT reporter construct

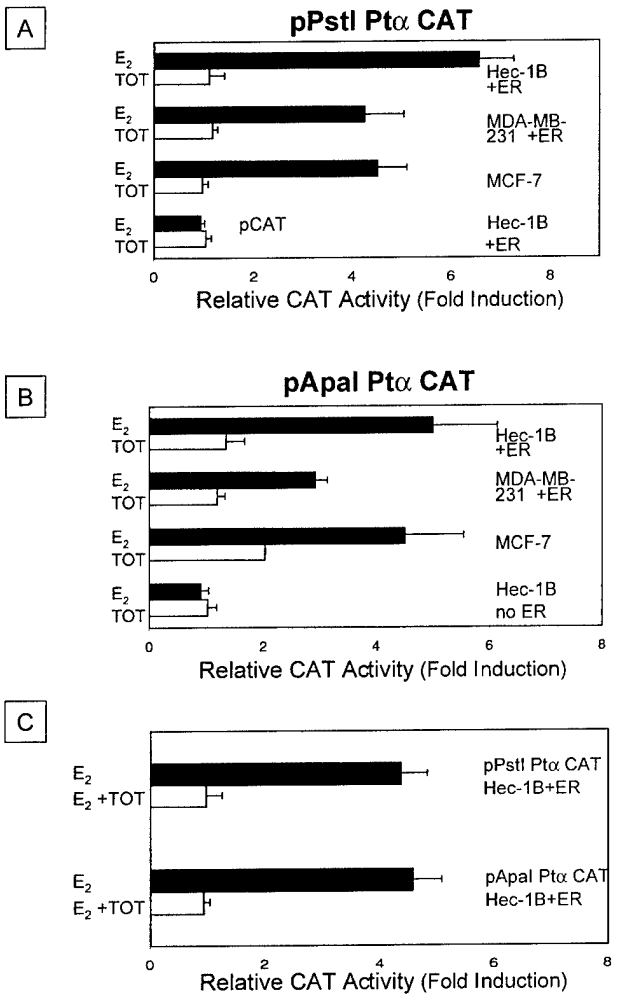

Figure 2 Activation of PTα CAT requires ERα. MDA-MB-231, MCF7 breast cancer cells and Hec-1B endometrial cancer cells were transfected with the PTα gene reporter construct, (**a**) PstI PTα CAT or (**b**) ApaI PTα CAT, along with an expression vector for the wild type human estrogen receptor. pCAT vector (lacking PTα promoter) and cmv5 vector (lacking ER cDNA) were used as controls. In (**c**) Hec-1B cells were transfected with ERα expression vector along with the PstI PTα CAT or ApaI PTα CAT reporter vectors. To normalize for transfection efficiency, cells were transfected with a β-galactosidase internal control reporter. Cells were then treated for 24 h with control ethanol vehicle (c), E_2 (10^{-8} M) and/or TOT (10^{-7} M) as indicated. Cell extracts were prepared and analysed for CAT activity and β-galactosidase activity. Values are the means ± s.e. from three or more separate experiments

among the three cell lines examined. Using several pApaI PTα CAT deletion mutants we identified a 260 bp fragment flanked by the *Sal*I (−722 bp) and *Sma*I (−460 bp) sites as important for estrogen induction of PTα promoter activity (Figure 3a). While deletion of the *Xho*I/*Sal*I fragment significantly increased basal reporter activity, E_2 was still able to induce reporter activity about fourfold over basal activity. When the fragment encompassed by *Sal*I/*Sma*I was broken into two parts, *Sal*I/*Sma*I (−0.72/−0.59 kb) *Sma*I/*Sma*I (−0.59/−0.46 kb), and introduced into the pCAT3 promoter vector, E_2-induced CAT reporter activity only in the *Sal*I/*Sma*I (−0.72/−0.59 kb) fragment (Figure 3b). The fold-induction from the pSalI/SmaI PTα CAT cannot be attributed to change in basal activity as this parameter was not significantly different among the pPstI PTα CAT, pApaI PTα CAT, and pSalI/SmaI PTα CAT reporter constructs.

The *Sal*I/*Sma*I (−0.72/−0.59 kb) region of the PTα gene was divided into three 43 bp fragments and oligonucleotides were synthesized corresponding to these fragments. Oligonucleotides were cloned into the pCAT3 reporter vector upstream of the heterologous SV40 promoter. The reporter constructs were transfected into Hec-1B cells along with an expression vector for ERα. Similar fold-inductions with E_2 were observed with the (−679/−637)-PTα CAT reporter constructs as that observed with the pPstI PTα CAT reporter (Figure 3b). No significant induction over basal activity by E_2 was evident with reporter constructs containing the other two fragments, −722/−680 and −636/−594. Of note, there were no differences in basal activity between the *Sal*I/*Sma*I fragment and each of the 43 bp fragments it contains.

Sequence analysis of the −679/−637 fragment indicates no consensus sequence for binding sites for any known transcription factors except for an incomplete half-ERE at −677 to −672 (Figure 3c). While there are reports of ER binding to half-EREs, there are also reports of binding of other transcription factors to the half-ERE that results in the regulation of ER-mediated gene transcription (Chen *et al.*, 1998; Garnier *et al.*, 1997; Klinge *et al.*, 1997). Also of interest are three repeats of a TGCCC element in this region, one next to the half-ERE and two sequentially ordered at the 3′ end (Figure 3c). To analyse the role of the incomplete half-ERE, these six residues were mutated, introduced into the pCAT3 vector (mut 1) and transfected into Hec-1B cells along with an expression vector for ERα. In these experiments we observe no activation of reporter activity upon mutation of the half-ERE (Figure 3c). Mutations introduced −659 to −639 (mut 3, 4 and 5) also disrupted E_2 fold-induction. The magnitude of response to estrogens did not change with mut 2, however a significant decrease in basal activity was observed resulting in a higher fold-induction relative to wild type PTα −679/−637 fragment. Our mutational analyses also indicate that the two TGCCC elements at the 3′ end are important for estrogen activation.

To further verify the importance of this novel estrogen response element, we introduced mutations 1 and 5 into the context of the natural PTα ApaI promoter. This would allow us to better assess the physiological relevance of this element on the natural promoter. When pApaI PTα pCAT (mut 1) and pApaI PTα pCAT (mut 5) were transfected into Hec-1B cells, the E_2 responsiveness was completely blocked with mut 5 but not mut 1 (Figure 3d). Thus the 3′ end of the PTα −679/−637 fragment is

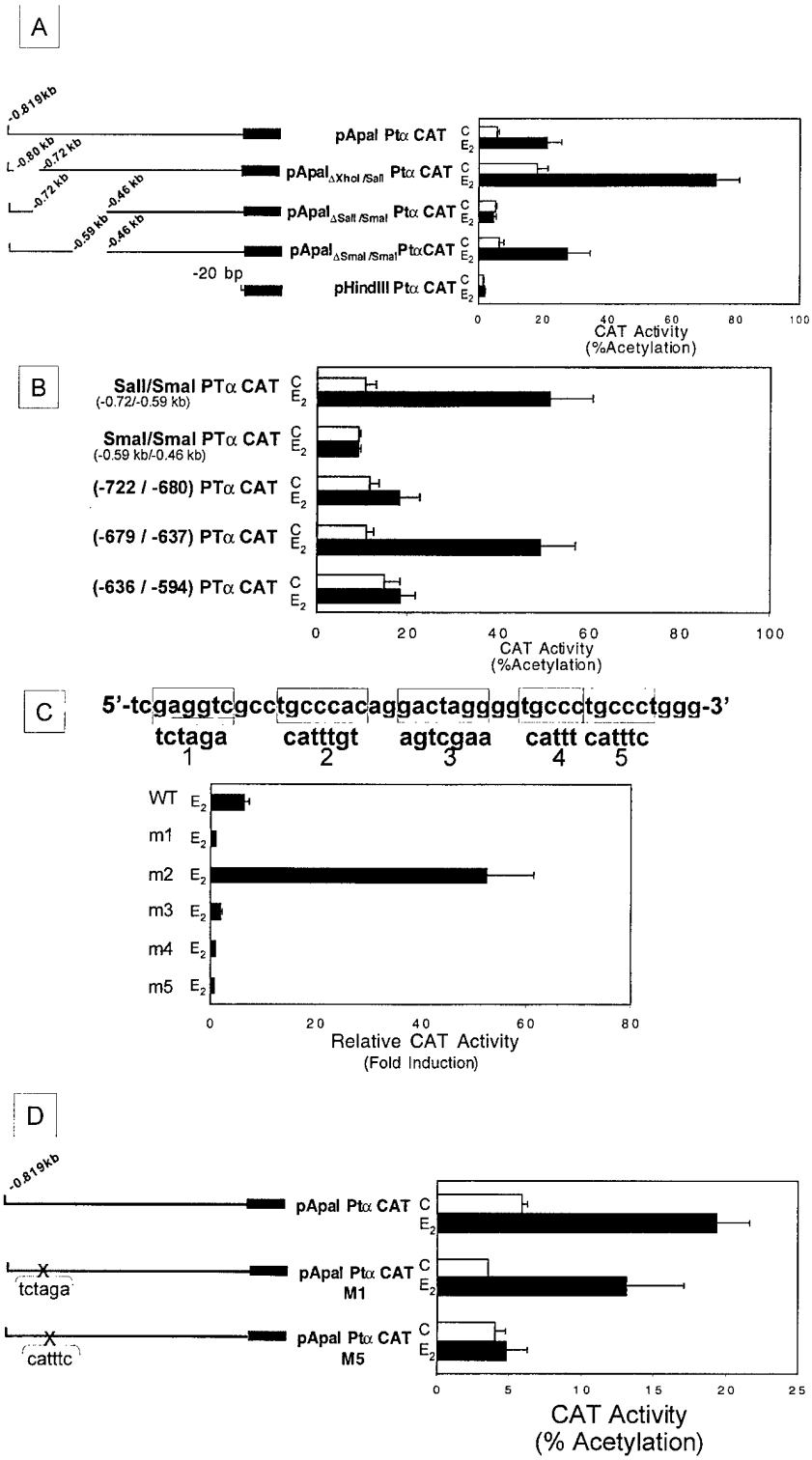

Figure 3 Identification of PTα gene promoter regions involved in activation by estrogens. (**a**) The PTα gene reporter construct, pApaI PTα CAT, or deletion mutants were transfected into Hec-1B cells along with an expression vector for the ERα. (**b**) PTα gene reporter constructs containing the region encompassed by *Sal*I/*Sma*I, *Sma*I/*Sma*I, or fragments of the *Sal*I/*Sma*I regions were transfected into Hec-1B cells along with an expression vector for the ERα. (**c**) Wild type and mutant (−679/−637)-PTα CAT reporter constructs were transfected into Hec-1B cells along with an expression vector for ERα. The half-ERE is underlined in the wild type sequence. The corresponding mutant is underneath the wild type sequence. (**d**) Wild type and mutant ApaI PTα CAT reporter constructs were transfected into Hec-1B cells along with an expression vector for ERα. In (**a**), (**b**), (**c**), and (**d**) cells were treated for 24 h with control ethanol vehicle (C) or E_2 (10^{-8} M) as indicated. Cell extracts were prepared and analysed for CAT activity and β-galactosidase activity. Each value represents the mean of three or more separate determinations ± s.e.m.

important for estrogen activation. While the incomplete half-ERE may be important for transcriptional activation in the context of a heterologous SV40 promoter, it does not appear to be necessary in the context of the natural promoter.

Identification of ERα functional domains involved in activation of PTα gene transcription

Further insight into the mechanism of ERα regulation of PTα gene transcriptional activation can be obtained from studies identifying the functional domains of the ERα required for PTα gene transcriptional activation. We examined the ability of ERα mutants with impaired activation function, hormone binding ability, or DNA binding ability to activate PTα gene reporter constructs. These experiments were conducted in Hec-1B cells where low levels of endogenous ERα allowed us to assess mutant ERα function. No activation of the (−679/−637)-PTα CAT reporter construct was observed with expression vectors for ERα with mutations in the DNA binding domain and AF2 region (Figure 4). ERα with three mutations in the DNA binding domain (HE82) that converts DNA binding specificity from an ERE to Glucocorticoid Receptor Response Element (GRE) (Mader *et al.*, 1989) did not induce enhancer activity. However deletion of the A/B domain, which has been shown to disrupt Activation Function 1 (AF-1) of the ER (Ali *et al.*, 1993) did not significantly affect the ability of ERα to mediate activation of the PTα gene promoter reporter construct. Our data suggest that the DNA binding and AF2 domains, but not the AF1 region, are required for transcriptional activation of PTα gene.

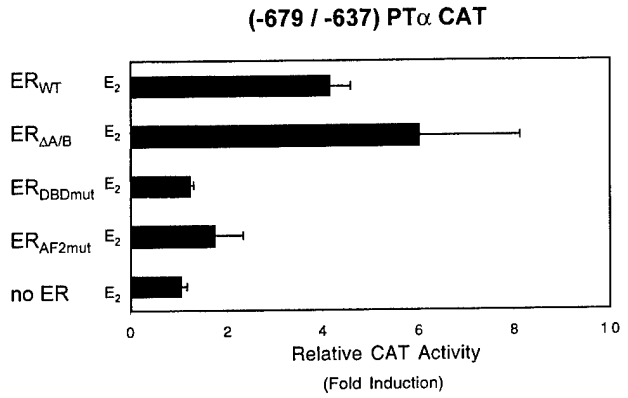

Figure 4 Identification of functional domains of ERα involved in the activation of PTα gene transcriptional activity. The PTα gene reporter construct, (−679/−637)-PTα CAT, along with expression vectors for wild type or mutant ERα were transfected into Hec-1B cells. Cells were then treated for 24 h with control ethanol vehicle (c) or E_2 (10^{-8} M) as indicated. Cell extracts were prepared and analysed for CAT activity and β-galactosidase activity. Each value represents the mean of three or more separate determinations ± s.e.m.

Biochemical analyses of interactions of MCF7 breast cancer cell factors with the Prothymosin α gene promoter region

Gel shift assays were then conducted to dissect the DNA-protein complex(es) that occur within the 43 bp region that mediate ERα regulation. ^{32}P end-labeled (−679/−637)-PTα oligonucleotides were incubated with purified recombinant ERα. A representative autoradiograph is shown in Figure 5 and indicates the presence of one major DNA-protein complex as a shifted band (SB). The specificity of the DNA-protein interactions was verified using competitive gel shift assays with unlabeled (−679/−637)-PTα oligonucleotide. The DNA-protein complex was supershifted by ERα antibody and could be competed off by unlabeled consensus ERE. Thus estrogen transcriptional activation appears to be mediated through ERα binding to PTα gene promoter elements. However, ERα binding to the PTα promoter element is much weaker than ERα binding to the ERE (Figure 5, lanes 11−12).

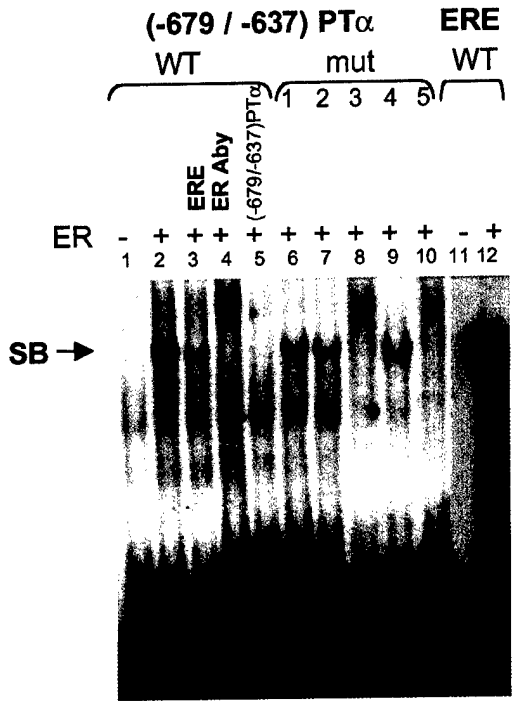

Figure 5 Identification of elements involved in ERα binding to PTα gene fragment (−679/−637). Gel mobility shift assays were performed using a double stranded oligomer containing the wild type or mutant −679/−637 region of the human PTα gene along with purified ERα protein. Wild type and mutant ^{32}P(−679/−637)-PTα were incubated with purified recombinant ERα (lanes 2−10) in the absence or presence of 100-fold excess of unlabeled ERE, 100-fold excess of unlabeled −679/−637 fragment, or monoclonal ERα antibody. In lanes 11 and 12, ^{32}P-ERE was incubated in the absence or presence of recombinant ERα, respectively. Equal c.p.m. and ng amounts of ^{32}P-(−679/−637)-PTα, ^{32}P-(−679/−637)mut-PTα, and ^{32}P-ERE or equal amounts of recombinant ERα were used in the binding reactions. The autoradiographs are representative of three separate experiments

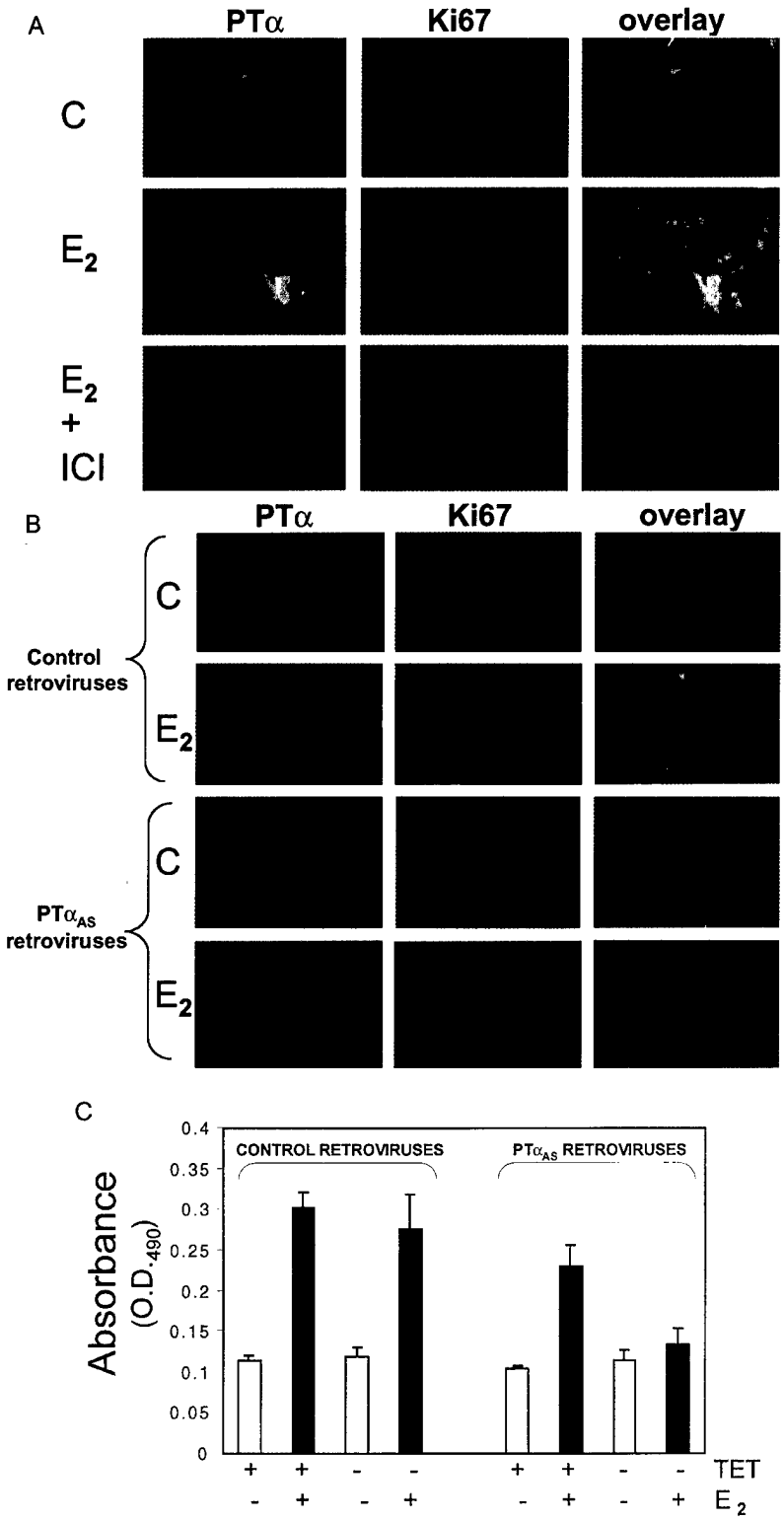

Figure 6 Inhibition of PTα expression attenuates E_2-induced breast cancer cell proliferation. (**a**) MCF7 cells were fixed for immunostaining 24 h after treatment with control ethanol vehicle (C), 10^{-8} M E_2, 10^{-8} M $E_2 + 10^{-7}$ M ICI 182,780 as indicated. In (**b**) and (**c**) MCF7 cells were infected with control or antisense PTα retroviruses. Two days after infection cells were treated with vehicle or 10^{-8} M E_2. (**b**) Cells were fixed for immunostaining 24 h later or (**c**) cell number was determined 5 days later using the CellTiter 96 Aqueous One Solution Proliferation Assay. Cells were viewed under a fluorescent microscope at 200 × magnification. Background staining, as measured using control IgG, was subtracted from images. Values for cell number are expressed relative to the absorbance in control cells grown in the presence of tetracycline (which is set at 1). Values are the means ± s.e. from two separate experiments with triplicate wells for each group

Further studies were conducted to determine the binding sites for ERα. Gel shift analyses indicate that ERα binding is disrupted upon mutations at 659/−653 (mut 3) and a TGCCC element at −645/−640 (mut 5) (Figure 5). These results are consistent with the involvement of these regions in ERα-mediated transcriptional activation. Mutation of the half-ERE at −677/672 did not disrupt ERα binding which is consistent with our observation that this element is not necessary for ERα activation in the context of the natural promoter. Mut 4, while not affecting ERα binding, is associated with decreased ERα-mediated activation. It is likely that this site may bind to accessory factors required for ERα-mediated activation.

Determination of the relevance of ERα regulation of PTα transcriptional activity on breast cancer growth

We then determined if estrogen-induced PTα expression can be correlated with increased proliferation. For these studies we used immunofluorescence staining to determine if the same cells that express PTα are also proliferating. PTα has been proposed to move between nuclear and cytoplasmic compartments (Enkemann *et al.*, 2000). However the relative importance of nuclear and cytoplasmic localization on its proliferative effects has not been examined. We used expression of Ki67 as a measure of proliferation status of the cells as well as a control for nuclear staining (Iatropaulos and Williams, 1996). Twenty-four hours after treatment, we see an increase in the number of cells that show primary nuclear (and decreased cytoplasmic) localization of PTα in the presence of estrogens, which was decreased when antiestrogen ICI182,780 was added (Figure 6a). Ki67 staining is highest in cells wherein PTα is primarily nuclear.

A self-contained tetracycline-regulated retroviral vector system (Paulus *et al.*, 1996) was used to express antisense PTα ($PT\alpha_{AS}$). In the presence of tetracycline, expression of $PT\alpha_{AS}$ is inhibited. An ensuing $25\pm7\%$ and $48\pm10\%$ decrease in PTα expression in control and E_2 treated cells, respectively, was observed in MCF7 cells after infection with $PT\alpha_{AS}$ retroviruses (Figure 6b). Infection with $PT\alpha_{AS}$ retroviruses decreased PTα nuclear staining and Ki67 expression normally observed 24 h after treatment with E_2. To confirm this, we also used a proliferation assay. While control cells show the expected increase in cell number after 6 days treatment with E_2, cells infected with $PT\alpha_{AS}$ retroviruses showed no increase in proliferation in the presence of E_2 (Figure 6c). These findings suggest that induction of PTα expression by E_2 plays a role in E_2-mediated breast cancer growth induction.

Discussion

In summary, we have shown estrogen-stimulated gene transcription from PTα gene promoter-containing reporter constructs. We have delimited the region required for estrogen-mediated induction to a 43 bp fragment, and the mechanism for ERα-mediated activation most likely involves a complex interplay between ERα and other protein factors bound to this region. The transcriptional activation by estrogens appears to involve ERα binding to the PTα gene promoter. Estrogen treatment also resulted in increased PTα nuclear localization which in turn is correlated with increased cell proliferation. Our studies also indicate that PTα plays a role in E_2-induced proliferation of breast cancer cells.

We observed PTα to be a gene upregulated in E_2-treated cells using Northern blot analyses. This increase was not observed with the antiestrogen TOT. Similarly Garnier *et al.*, using differential display report that PTα mRNA expression was also enhanced in neuroblastoma cells after estrogen treatment (Garnier *et al.*, 1997). The present studies now show that the increase in PTα mRNA in the presence of E_2 is also evident at the protein level. This protein has been of considerable interest to us due to its role in cellular proliferation. It is a highly acidic nuclear protein widely expressed in all cell types. While the exact mechanism of action of PTα remains elusive, the one constant has been its requirement for cellular growth and survival (Eschenfeldt and Berger, 1986; Gomez-Marquez *et al.*, 1989; Rodriguez *et al.*, 1998; Sburlati *et al.*, 1991; Wu *et al.*, 1997). Recently, PTα was shown to be capable of transforming rodent fibroblast cells in a manner similar to Ras, suggesting that PTα may be an important downstream target for inducers of cellular transformation (Orre *et al.*, 2001). PTα can serve as a marker for both breast cancer and hepatocarcinomas, and several malignant tissues have increased levels of PTα (Magdalena *et al.*, 2000; Pineiro *et al.*, 2000; Tsitsilonis *et al.*, 1998). Another source of interest for us is that PTα has also been shown to enhance ER transcriptional activity (Martini *et al.*, 2000), further validating its connection to estrogens.

Regarding the transcriptional regulation of PTα, there are still many unanswered questions. PTα mRNA levels are increased after serum restitution or after stimulation with various mitogens, further supporting its role in the cell cycle (Pineiro *et al.*, 2000; Zalvide *et al.*, 1992). However, levels of PTα do not vary significantly during the cell cycle making it hard to assign it to a particular cellular process (Pineiro *et al.*, 2000). While there is an E2F binding site between −323 and −316, the role of this site in E2F-induction of PTα gene transcriptional regulation has not been specifically tested (Eilers *et al.*, 1991; Szabo *et al.*, 1993). Our results suggest that this site is not necessary for ERα regulation. While the E-box at −1.173 kb of the PTα gene promoter has been proposed to mediate c-*myc* induction, c-*myc* activation of PTα expression has been put into doubt (Desbarats *et al.*, 1996; Gaubatz *et al.*, 1994; Mol *et al.*, 1995). Studies with antisense c-*myc* suggest that PTα expression is not completely dependent on c-*myc*. While c-*myc* is a well-known ER target gene (Dubik and Shiu, 1988) the present studies indicate that it is unlikely that E_2

upregulation of PTα occurs indirectly through E_2-mediated induction of c-*myc* expression. Our studies suggest that intervening protein synthesis is not required for the increase in PTα mRNA expression in the presence of E_2. Moreover, deletion of the E-box did not affect E_2-mediated upregulation of PTα gene transcriptional activity. Another group has shown that E_2 does not regulate the expression of N-*myc*, the neural counterpart of c-*myc* in neural cells, wherein E_2 has been demonstrated to upregulate PTα expression (Garnier *et al.*, 1997). Additional supporting evidence for direct transcriptional regulation of the PTα gene by the ER is that the increase in PTα expression was observed 1 h after estrogen treatment in neuroblastoma cells (Garnier *et al.*, 1997).

While there are no full EREs for binding of the ERα in the PTα promoter, there are several half-palindromic EREs that may be able to promote transcription (Chen *et al.*, 1998; Klinge *et al.*, 1997). However, the incomplete half-ERE (AGGTCG) in the 43 bp region does not appear to be essential for ERα regulation of PTα gene promoter or for ERα binding. The 3′ end of the 43 bp region important for ERα transcriptional activation and binding does contain a direct TGCCC (or GCCCT) repeat. Since a GCCCT direct repeat has been shown to bind Sp1 (Dennig *et al.*, 1995) and Sp1 can interact with ERα to transactivate genes (Safe, 2001), we investigated the possibility that this element may recruit ERα through Sp1 interaction. However, gel shift analysis revealed no binding of Sp1 to this element or significant effects on ERα activation (data not shown).

During the preparation of this manuscript, there was a report from another group examining the regulation of PTα by estrogens (Martini and Katzenellenbogen, 2001). Their studies indicate the involvement of two half-EREs in the regulation by estrogens. These half-EREs, located at −886 to −861 and −588 to −560, were also proposed (but not examined) by Garnier *et al.* (1997) to be involved in estrogen regulation of PTα expression in neuroblastoma cells. While mutational analyses support the involvement of these two half-EREs (Martini and Katzenellenbogen, 2001), the half-EREs were not cloned upstream of a heterologous promoter to indicate enhancer activity. Our results indicate that when the *Sma*I/*Sma*I region (containing the half-EREs at −588 to −560) was cloned upstream of a heterologous promoter no E_2-mediated activation was evident. In addition when the half-ERE at −886 to −861 was deleted (compare ApaI PTα CAT with PstI PTα CAT) we did not see a significant decrease in E_2-mediated activation. Thus the decrease in promoter activity resulting from mutation of the two half-EREs may be attributed to disruption of intermolecular interactions in the promoter region, rather than promoter context-independent transcriptional regulation. The differences between these two reports may be also attributed to methodological differences.

Overall our findings reveal an interesting cross-talk between ERα and PTα. As mentioned above it has been shown that PTα can influence ER transcriptional activity (Martini *et al.*, 2000). PTα does not directly interact with ER but appears to sequester the anti-coactivator factor REA from the ER transcriptional complex. ER is then able to interact with its coactivators. Certain aspects of PTα structure may be useful in understanding the transcriptional regulatory function of PTα. Because of the high acidity of the protein (Palvimo and Linnala-Kankkunen, 1990) it is unlikely to be a DNA binding protein. There are structural similarities between PTα and nuclear proteins known to be involved in chromatin activity, and it has been reported that PTα interacts specifically with histone H1 (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsou *et al.*, 1998). PTα is proposed to play a role in nucleosome assembly (Diaz-Jullien *et al.*, 1996; Gomez-Marquez and Rodriguez, 1998; Karetsou *et al.*, 1998), implicating a putative nuclear function related to chromatin remodeling. Transcriptional studies suggest a role for histone H1 in the high compactation of DNA and hence the general repression of transcription; other studies suggest H1 to be involved in the transcriptional repression of a selected group of genes (reviewed in Crane-Robinson, 1999; Wolfe *et al.*, 1997) PTα may play a role in transcriptional activation by associating with histone H1 and releasing histone H1 from chromatin. By functioning as a histone receptor (histone sink), PTα may allow access of the basal transcription factors to the DNA template. Regardless of the exact mechanism of PTα regulation of ER transcriptional activity, the regulation of PTα expression by E_2 adds another level of complexity and may represent a positive feedback loop for E_2-ERα regulation of transcription.

Our studies suggest that estrogens may be involved in the regulation of PTα localization. While the localization of PTα have been initially reported to be primarily nuclear, more recent reports also indicate cytoplasmic localization. However, none of these studies have compared PTα localization in the absence and presence of mitogenic agents. We observe an increase in PTα nuclear localization in the presence of estrogens which translates to increased cell proliferation. Conversely, infection with $PT\alpha_{AS}$ retroviruses results in decreased estrogen-induced nuclear PTα localization and decreased cell proliferation. These findings also suggest that the increase in PTα protein expression in the nucleus may be attributed to the transcriptional effects of estrogen on PTα gene transcription.

While the exact details of the mechanism of action of PTα remain elusive, it is clearly involved in the regulation of cell transformation and proliferation. The estrogen receptor (ER) is a ligand activated transcription factor and the identification of 'primary' ER target genes is imperative for understanding the basis for the proliferative action of ER in breast cancer cells. There are only a few candidate genes that appear to be under the direct regulation of the ER; much less genes that are associated with cell proliferative activity. PTα is a particularly strong candidate because its

expression and intracellular localization appears to be regulated by estrogen, and down-regulation of PTα expression inhibits E_2-induced breast cancer cell proliferation.

Materials and methods

Chemicals and materials

Cell culture media was purchased from GIBCO (Grand Island, NY, USA). Calf serum was from Hyclone Laboratories (Logan, UT, USA) and fetal calf serum from Sigma Chemical Company (St. Louis, MO, USA). 17β-Estradiol (E_2), trans-hyroxytamoxifen (TOT), and cycloheximide (CHX) was obtained from Sigma Chemical Company. ICI182,780 was obtained from Tocris (Ballwin, MO, USA). Custom oligonucleotides were purchased from Genosys (Grand Island, NY, USA).

Northern blot analyses

Total RNA was isolated using Trizol (GIBCO–BRL, Rockville, MD, USA). Gel purified reamplified PTα cDNA was random primer labeled using the Ready-to-Go DNA labeling kit from Pharmacia (Piscataway, NJ, USA) for Northern analysis. Twenty μg of total RNA was separated by electrophoresis, transferred to nitrocellulose support and hybridized with random primer labeled cDNA (Cho *et al.*, 1991). Full-length cDNA for human PTα was obtained from ATCC (Manassas, VA, USA). Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Western blot analyses

Whole cell extracts were prepared from breast epithelial MCF7 cells as previously described (Wrenn and Katzenellenbogen, 1993). Proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels and transferred electrophoretically onto 0.2 micron nitrocellulose membranes. Blots were incubated with anti-PTα polyclonal antibody (1 : 2000 dilution, ImmunDiagnostik, Bensheim, Germany) and goat anti-rabbit IgG secondary antibody (1 : 30 000 dilution) for detection by chemiluminescence (Super Signal West Femto, Pierce, Rockford, IL, USA). Quantitative analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Plasmid construction and mutagenesis

All cloning was done using standard techniques (Ausubel *et al.*, 1992; Sambrook *et al.*, 1989). The reporter vectors pPstI PTα CAT (containing the 5′ regulatory region of the PTα gene from the *Pst*I site located −5 kb from the transcriptional start site) and pApaI PTα CAT (containing the 5′ regulatory region of the PTα gene from the *Apa*I site located −0.819 kb from the transcriptional start site) were obtained from Dr Shelby Berger (National Cancer Institute, Bethesda, MD, USA). Deletions of the pApaI PTα CAT constructs were constructed using the available restriction sites.

Reporter constructs containing fragments of the *Sal*I/*Sma*I region were constructed using the following oligonucleotides with their complement: PTα (722–680): 5′-cgcgtcgactgacttggccgacggacgccggacctgatggtgggcaa-3′ PTα (679–637): 5′-cgcgtcgaggtcgcctgcccacaggactaggggtgccctgccctggg-3′ PTα (636–594): 5′-cgcgaggggcgagggcatggcagagggacggggcggcccaggtgccc-3′ Each oligonucleotide and their complement were annealed, gel purified and cloned into the *Mlu*I/*Xho*I-digested pCAT3 promoter vector (Promega, Madison, WI, USA). Oligonucleotides containing mutations: PTα (679–637)mut 1: 5′-cgcgtctctagagcctgcccacaggactaggggtgccctgccctggg-3′ PTα (679–637)mut 2: 5′-cgcgtcgaggtcgcccatttgtaggactaggggtgccctgccctggg-3′ PTα (679–637)mut 3: 5′-cgcgtcgaggtcgcctgcccacagagtcgaaggtgccctgccctggg-3′ PTα (679–637)mut 4: 5′-cgcgtcgaggtcgcctgcccacaggactaggggcattttgccctggg-3′ PTα (679–637)mut 5: 5′-cgcgtcgaggtcgcctgcccacaggactaggggtgccccatttcagg-3′ were annealed to their complement, gel purified, and cloned into *Mlu*I/*Xho*I-digested pCAT3 promoter vector. The first four nucleotides from the 5′ end of the oligonucleotides were added for cloning into the pCAT3 vector.

The expression vectors for the wild type human ERα (pCMV5-ER), ERα Activation function mutant $ER\alpha_{AA/B}$, ERα DNA binding mutant ER HE82 (E203G/G204S/A207V) which changes DNA binding specificity to a GRE, and ERα Activation Function 2 mutant ER_{AF2mut} (L540Q/E542A/D545A) have been described previously (LeGoff *et al.*, 1994; Montano and Katzenellenbogen, 1997; Montano *et al.*, 1996; Wrenn and Katzenellenbogen, 1993; Mader *et al.*, 1989). The plasmid pCMVβ (Clontech, Palo Alto, CA, USA) which encodes the β-galactosidase gene, was used as an internal control for transfection efficiency in all experiments.

Cell culture and transfections

MCF7, MDA-MB-231 and Hec-1B cells were maintained and transfected as previously described (Montano and Katzenellenbogen, 1997). Cells were seeded for transfection in 100-mm dish in Improved Minimum Essential Media (IMEM) minus phenol red containing 5% CDCS. Cells were transfected as previously described (Montano *et al.*, 1996), and using 2 μg of PTα gene promoter reporter constructs, 10 ng of ERα expression vector, and 0.2 μg pCMVβ-galactosidase internal control plasmid. β-galactosidase activity, which was measured to normalize for transfection efficiency, and CAT activity were assayed as previously described (Montano *et al.*, 1996).

Gel shift assays

Human recombinant ERα was obtained from PanVera Corp. (Madison, WI, USA). The single stranded oligomers, representing wild type and mutant PTα (679–637) were annealed to their complement oligonucleotides. Double stranded oligomers were gel purified on a nondenaturing 4.5% polyacrylamide gel run in 1×TBE. The ability of purified protein to bind to the PTα gene promoter fragments was analysed using standard gel mobility shift assays (Montano *et al.*, 2000). Briefly 700 fmol of recombinant ERα was mixed with 1 ng of end-labeled PTα gene oligomer in the presence of 0.4 μg/μl dIdC, 20 mM HEPES (pH 7.9), 200 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 2 mM EDTA, 20% glycerol, 1 μg/μl BSA and incubated at room temperature for 20 min. The specificity of binding was assessed by competition with excess unlabeled double stranded PTα gene fragment. The presence of ERα in DNA-protein complexes was verified using supershift assays with ERα antibody, H222 (Abbott Laboratories, Chicago, IL, USA). The non-denaturing gels used to analyse the protein-DNA complexes were run as described previously (Montano *et al.*, 2000).

Retroviral-mediated transfection and immunostaining

Retroviruses were made by transfecting PA317 cells using the $CaPO_4$ coprecipitation method with the pBPSTR1 plasmid alone (to make control retroviruses) or pBPSTR1 containing PTα cDNA in the antisense orientation (Paulus *et al.*, 1996). PA317 media containing retroviruses was collected 48 h later and passed through a 0.45 micron filter. Breast epithelial cell lines were infected with retrovirus-containing supernatants in the presence or absence of 3 μg/ml tetracycline. When tetracycline was added, expression of the viral gene was inhibited.

PTα protein expression was examined by immunostaining using PTα antibody. Cells infected with control or antisense PTα retroviruses were grown on coverslips and subsequently fixed in 4% paraformaldehyde. After blocking with serum, samples were incubated with anti-PTα polyclonal antibody and goat, anti-rabbit IgG Alexa 488 fluorescence secondary antibody. As a negative control duplicate sections were immunostained with nonspecific rabbit IgG. Proliferating cells were identified by immunostaining using Ki67 IgG monoclonal antibody (Lab Vision) and goat, anti-mouse Alexa 594 secondary antibody (Molecular Probes). Semi-quantitative analysis was performed on a Macintosh computer using Adobe Photoshop 6.0 software. Mean luminosity of 20 cells from three separate experiments was measured and averaged with background subtracted out from each field.

Proliferation assays

1×10^5 cells were seeded in a 24 well plate. Two days after plating, fresh media was added containing hormones. Fresh media with hormones was added every two days. Cell number was determined five days after initial hormone treatment using the CellTiter 96 Aqueous One Solution Proliferation Assay (Promega, Madison, WI).

Abbreviations

PTα, prothymosin α; ERα, Estrogen Receptor α; E_2, estradiol; TOT, trans-hydroxytamoxifen; ERE, estrogen response element

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